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The AIDS Disease of CD4C/HIV Transgenic Mice Shows Impaired Germinal Centers and Autoantibodies and Develops in the Absence of IFN-γ and IL-6

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Summary

The mechanisms responsible for degeneration of germinal centers (GC) and follicular dendritic cell (FDC) networks during progression to AIDS remain elusive. Here, we show that CD4$^+$ T cells from CD4C/HIV-1 Tg mice, which develop a severe AIDS-like disease, express low levels of CD40 ligand. Accordingly, GC formation, FDC networks, and immunoglobulin isotype switching are impaired in these animals. However, Tg B cells respond to in vitro CD40 stimulation. Total serum IgG levels are reduced in Tg mice, whereas total IgM levels are increased with a significant amount showing DNA specificity. IFN-γ- and IL-6-deficient CD4C/HIV Tg mice also develop the AIDS-like disease and produce auto-Ab. Thus, CD4C/HIV Tg mice have immune dysfunction accompanied by autoimmune responses.

Introduction

We have previously reported that CD4C/HIV-1 Tg mice develop a severe disease closely resembling pediatric AIDS (Hanna et al., 1998a, 1998b). These mice die prematurely, suffering from severe wasting as well as kidney, lung, and heart pathologies. A gradual atrophy of the lymphoid organs also occurs with preferential loss of CD4$^+$ T cells, which is accompanied by a downregulation of the CD4 molecule and a loss of responsiveness to mitogenic stimuli. In contrast, B cell numbers are increased in Tg lymph nodes (LN) and spleens.

Expression of HIV-1 in Tg mice is under the control of the transcriptional regulatory elements of the human CD4 gene and has thus been detected in immature and mature CD4$^+$ T cells as well as in cells of the macrophage/dendritic cell lineages (Hanna et al., 1998a, 1998b). Studies with several HIV-1 mutant Tg lines revealed that *nef* was largely responsible for the development of the AIDS-like pathology (Hanna et al., 1998b).

GC develop in the primary follicles of secondary lymphoid organs during T-dependent B cell responses. They are sites in which maturation and selection of high-affinity antibodies (Ab) occur, and are necessary for the generation and maintenance of immunological memory (MacLennan, 1994; Kosco-Vilbois et al., 1997). The selection of high-affinity Ab-bearing B cells involves signals from CD4$^+$ T cells as well as from FDC, the latter maintaining immune complexes on their surface through complement and Fc receptors. Signals from FDC are important for the establishment of GC, regulating lymphocyte migration to the follicle and access to antigens (Ag). It has been widely demonstrated that T-dependent immunoglobulin (Ig) isotype switching and GC reactions leading to immunological memory are mainly dependent on CD40 ligand (CD40L)-mediated T cell help for B cell activation and on T helper cell cytokine production. IL-6 production by FDC has also been demonstrated to promote GC development and Ab production (Kopf et al., 1998), and IL-6 has been shown to be involved in B cell terminal differentiation (Morse et al., 1997).

Lymphoid tissues are the main reservoir for HIV-1, where ongoing cellular interactions allow for the virus to be produced and persist. More precisely, the GC compartment comprises the major cellular source for viral replication and storage (Pantaleo et al., 1993; Pantaleo and Fauci, 1995; Embretson et al., 1993; Fox et al., 1991; Tenner-Racz and Racz, 1995). Indeed, activated/memory CD4$^+$ T cells, which traffic to GC, are the major cells involved in viral replication and production. Also, although not infected with HIV-1, FDC act as a significant storage compartment for virions (Pantaleo and Fauci, 1995; Tenner-Racz and Racz, 1995).

Dysfunctional GC formation is a hallmark of SIV- and HIV-1-mediated diseases (Pantaleo et al., 1993; Pantaleo and Fauci, 1995; Rosenberg et al., 1997). Progression to AIDS is associated with the disruption and involution of the GC architecture concomitant with the loss of virion trapping capacity in lymphoid organs due to the degeneration of FDC networks. Whether the latter results from cytopathic effects remains to be established. However, given that FDC network formation depends on both B and T cell signals (MacLennan, 1994; Kosco-Vilbois et al., 1997), the gradual loss of T cells and immune dysfunctions associated with AIDS are likely to contribute to GC involution and FDC network destruction.

The B cell compartment is most probably affected by HIV-1 through an indirect mechanism, as the virus has not been shown to infect or replicate in B cells in vivo (Shearer, 1998; Edelman and Zolla-Pazner, 1989). B cell hyperreactivity and auto-Ab production are often reported in AIDS patients (Pantaleo and Fauci, 1995; Shearer, 1998; Root-Bernstein, 1990; Ditzel et al., 1994). In addition, B cell lymphomas develop at a relatively high frequency during AIDS (Koopman and Pals, 1992;

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Herndier et al., 1994). These pathogeneses involve cytokines such as IL-6, IL-10, and IL-13 (Emilie et al., 1997).

In order to understand the effects of HIV-1 proteins on $CD4^+$ T helper functions in relation to B cell responses, we have studied the lymphoid compartment of CD4C/HIV-1 Tg mice, assessing their capacity to generate GC reactions and develop FDC networks. In addition, we have investigated whether the cytokines IFN-γ and IL-6, known to be involved in T and B cell functions, were required for the development of the AIDS-like disease.

Results

Phenotype of $CD4^+$ T Cells from CD4C/HIV^{MutA} Tg Mice

HIV-1 infection is characterized by the loss in number and function of $CD4^+$ T cells, as well as a downregulation of their CD4 surface expression (Shearer, 1998). In CD4C/HIV Tg mice, $CD4^+$ T cells are also preferentially and progressively lost during the course of the disease and exhibit a downregulation of CD4 surface expression (Hanna et al., 1998b; Figure 1A). Based on these observations, the function of $CD4^+$ T cells was further investigated in the CD4C/HIV^{MutA} Tg mice, expressing *rev*, *env*, and *nef* of HIV-1. Using flow cytometry (FACS), lower levels of the costimulatory molecule CD40L were found to be expressed in Tg $CD4^+$ T cells as compared to non-Tg $CD4^+$ T cells, following in vitro stimulation with anti-CD3 plus anti-CD28 mAb. However, CD69 expression was upregulated from 10% to 94% by non-Tg T cells and from 25% to 87% by Tg T cells (Figure 1B), and similar numbers of IL-2-producing $CD4^+$ T cells were detected by intracellular staining in cultures from non-Tg and Tg mice (X.W., unpublished data), suggesting that Tg $CD4^+$ T cells can respond to a certain extent to in vitro stimulation.

The capacity to produce cytokines of a differentiated helper phenotype was also assessed. After 2 days of stimulation with anti-CD3 plus anti-CD28 mAb, the number of IFN-γ-producing $CD4^+$ T cells was observed to be higher in cultures from Tg mice, whereas IL-4-producing $CD4^+$ T cells could not be detected in either cell population (data not shown). In order to further assess the capacity of Tg $CD4^+$ T cells to produce IL-4, T cells were first cultured for 5 days with anti-CD3 plus anti-CD28 mAb and restimulated in the same conditions for another 3 days, as described previously (Poudrier et al., 1998). Under these conditions, Tg cultures showed significant cell death and the remaining $CD4^+$ T cells did not produce IL-4, but a higher proportion of IFN-γ-producing Tg $CD4^+$ T cells was observed, as compared to non-Tg $CD4^+$ T cells (Figure 1C). FACS analysis using the DX5 mAb revealed the absence of NK cells in the $CD4^+$ T cell cultures (data not shown), ruling out a possible contribution of NK cells to the IFN-γ production. Interestingly, this profile of high levels of IFN-γ and low levels of IL-4 observed in CD4C/HIV^{MutA} Tg mice reflects what is found in human AIDS (Graziosi et al., 1994; Than et al., 1997; Emilie et al., 1994).

GC Formation Is Impaired in Tg Mice

The decreased level of CD40L on Tg $CD4^+$ T cells as well as the reduced capacity to produce IL-4 suggested that the delivery of T helper functions for B cell activation might be impaired. To further address this issue, the influence of $CD4^+$ T cell help on B cell activation in vivo was assessed by measuring the immune response to chicken ovalbumin (OVA). A paucity in GC formation in response to OVA was observed in draining LN of immunized Tg mice (Figure 2). The formation of peanut agglutinin (PNA) binding GC, observed in non-Tg mice sacrificed 7 days following the primary immunization, was largely absent from Tg animals (Figure 2B). In addition, the FDC network was markedly reduced in Tg animals, as compared to the non-Tg controls (Figure 2C). Also, the number of large IgM^+ B cells, possibly representing plasma cells, appeared to be increased in the Tg LN (Figures 2B and 2C). Similar findings were obtained in LN from Tg mice 10 days following primary (i.e., at the peak of GC reactions; Figure 2A) or 5 days following secondary (data not shown) immunizations with OVA, although a few small foci of PNA binding cells could be observed in the follicles of some Tg LN. In situ hybridization (ISH) performed on LN sections from the Tg mice revealed that HIV transgene-expressing cells were concentrated mainly in the T cell zone and subcapsular sinus (Figure 2I).

In the spleens, an absence of PNA binding GC was also observed in the Tg mice following OVA immunization (Figures 2D and 2E). The T cell zone was markedly decreased in the sections from Tg as compared to non-Tg animals (Figures 2D–2F). An enlarged marginal zone (MZ) could be observed in Tg mice (Figures 2D–2F) with numerous Mac-1^+ macrophages (Figure 2F), and strong PNA binding was detected on large cells of this MZ area (Figures 2D and 2E), including on $CD11c^+$ as well as Moma-1^+ cells (data not shown). In addition, evaluation at higher magnification revealed that more large IgM^+ B cells, possibly plasma cells, were found in the red pulp of Tg mice than in non-Tg controls, often surrounded by large PNA binding cells (Figure 2E). Interestingly, the MZ corresponded to the splenic area where high levels of HIV-1 expression were found by ISH along with the T cell zone (Figure 2M). Analysis of the spleen also revealed follicular hyperplasia in some Tg animals (Figures 2M and 2O).

Strikingly, strong PNA binding whose intensity was comparable to that obtained for GC cells (Figures 2A, 2B, and 3A) and for the thymus of both non-Tg and Tg animals (Figure 3D) was observed on cells of the T cell zone of both LN and spleen in Tg mice (Figures 2A and 3A–3C), regardless of whether the mice had been immunized (Figure 3). Some of these PNA^+ cells were $CD8^+$ (as assessed by double labeling; data not shown). However, the downregulation of CD4 prevented the obtaining of similar data for $CD4^+$ T cells. Nevertheless, those PNA^+ cells are most probably T cells, as most of the cells in the Tg T zone are TcR^+ (data not shown). PNA binding does not appear to involve B cells, as there was an absence of PNA binding by IgM^+, IgD^+, or $B220^+$ cells (data not shown). PNA binding cells were also prevalent in the MZ of the Tg spleen (Figures 2D and 2E) and LN subcapsular sinuses (Figures 2A, 2B, and 3A), although for this latter site less frequently than in the MZ. Strong PNA binding therefore appears to follow the pattern of transgene expression in LN and spleen of Tg mice. Although the T cell zone in Tg mice is diminished

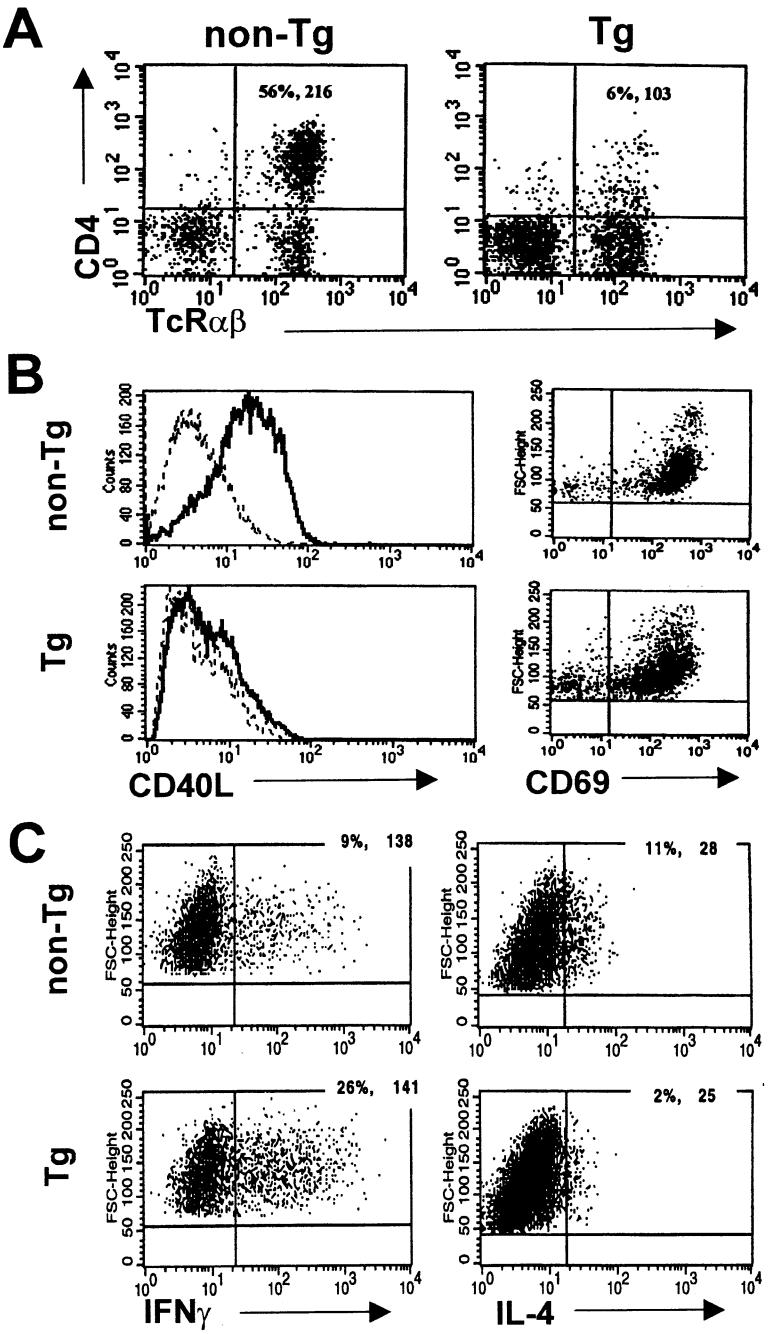

Figure 1. Phenotype of Tg CD4$^+$ T Cells

(A) FACS analysis of CD4 expression on total peripheral LN cells from non-Tg and Tg mice (4-month-old). The percentage of CD4$^+$ cells and mean fluorescence intensity are shown. The same observations were found for spleen and mesenteric LN. The results showing CD4 downregulation and reduction of CD4$^+$ T cells, to various degrees depending on the age of the animal, are representative of 12 experiments, analyzing mice of 1.5 to 4 months in age.

(B–C) High-density CD4$^+$ T cells from peripheral LN of non-Tg and Tg mice (2-month-old).

(B) Expression of CD40L and CD69 following a 12-hr culture period on anti-CD3-coated plates in the presence of anti-CD28 mAb.

(C) IFN-γ and IL-4 expression was detected by intracellular staining after an initial 5-day stimulation on anti-CD3-coated plates in the presence of anti-CD28 mAb, followed by a 3-day restimulation under the same conditions. The percentage of cytokine-positive cells and mean fluorescence intensity are shown. The data are representative of at least four experiments.

in size, it consists mainly of TcR$^+$ cells as well as of a few MHC class II$^+$ cells (data not shown) and IgM$^+$ B cells (Figures 2A and 3B), reflecting a normal cellular distribution (Steinman et al., 1997). Only in very severe disease states did we observe a prevalence of B cells in the T cell zones of Tg mice (data not shown).

GC Formation Is Not Impaired in Tg Mice Harboring a Mutated Nef Allele

It could be argued that the downregulation of CD4 on T cells may be contributing to the lowered T helper functions found in these Tg mice and consequently to some of the pathological changes observed. However, we found that Tg mice expressing a myristylation-negative mutant Nef allele (CD4C/HIV-Nef^{Myr-}) did not develop an AIDS-like disease (Z.H., unpublished data), despite the fact that they still exhibit a downregulation of CD4 on the surface of their T cells which is equivalent to that observed with wild-type Nef (30%–50% decrease in mean fluorescence intensity). Interestingly, these CD4C/HIV-Nef^{Myr-} mice can develop normal GC in response to immunization with OVA both in LN (Figure 2G) and spleen (data not shown). These results strongly suggest that mechanisms other than CD4 downregulation alone are responsible for the phenotype observed in the CD4C/HIV^{MutA} Tg mice.

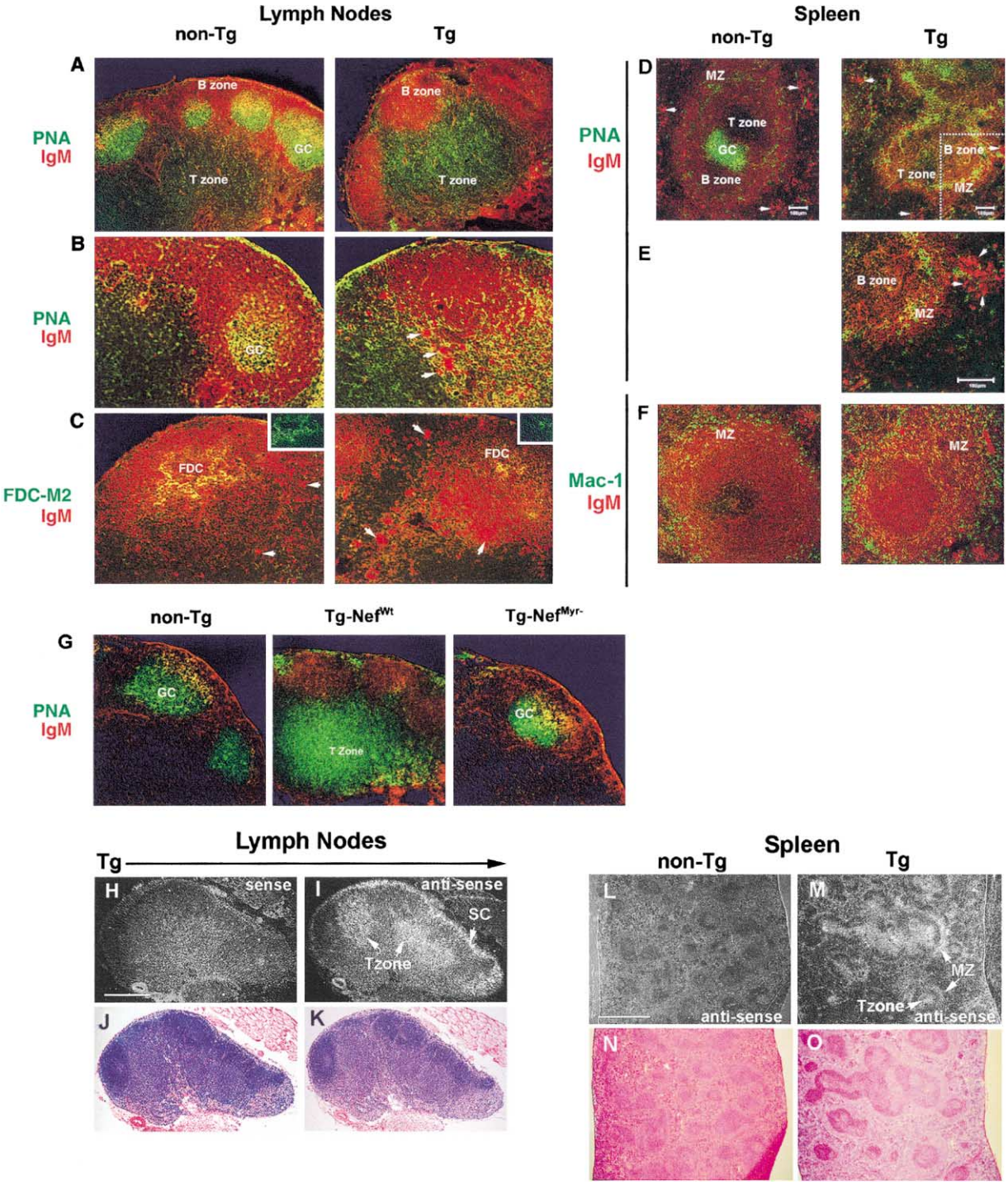

Figure 2. Impaired GC Formation and Transgene Expression in LN and Spleen of Tg Mice

(A–C) The formation of GC assessed on cryosections of draining LN taken at day 10 (A) or day 7 (B and C) following primary immunization with OVA. PNA binding is in green and IgM expression is in red. Magnification, 10× (A) and 40× (B and C). IgM expression is shown in red and the FDC network in green (C). Coexpression is in yellow. Insets in (C) highlight the extent of FDC labeling.

(D–F) The formation of GC assessed on splenic cryosections obtained at day 10 following a primary immunization with OVA. PNA binding (D and E) and Mac-1 (F) are in green and IgM expression is in red. A higher magnification (20×) of the dotted portion of (D) (10×) is shown in (E). Sections were analyzed by fluorescence (A–C and F) or confocal (D and E) microscopy. Data are representative of at least four experiments (16 mice). Arrows point to large IgM^+ cells (B–E).

(G) The formation of GC was assessed on cryosections of draining LN from non-Tg, CD4C/HIV^{MutG} (Tg-Nef^{WT}), and CD4C/HIV-Nef^{Myr-} (Tg-Nef^{Myr-}) mice, taken at day 10 following primary immunization with OVA. Analysis by confocal microscopy shows PNA binding in green and IgM expression in red. Magnification, 10×.

(H–O) Transgene expression in LN (I) and spleen (M) of CD4C/HIV^{MutA} Tg mice. Draining LN from an OVA-challenged Tg mouse was hybridized

Ig Isotype Switching Is Impaired in Tg Mice

Ig isotype switching occurs due to T cell-dependent B cell responses during GC reactions. Accordingly, the levels of various Ig isotypes in the sera of mice following primary (data not shown) and secondary (Figure 4A) immunizations with OVA were measured. Significantly reduced levels of OVA-specific IgG_1, IgG_{2a}, and IgG_{2b} were observed in the sera of Tg mice as compared to levels in non-Tg controls. The production of OVA-specific IgM was less affected, although most Tg animals had significantly lower levels than non-Tg mice. These results indicate that B cells from CD4C/HIV^{MutA} Tg mice have a reduced capacity to mount a mature Ag-specific Ab response. Interestingly, this capacity to Ig isotype switch was maintained in the CD4C/HIV- Nef^{Myr-} Tg mice (data not shown).

High Level of Serum IgM and Presence of Anti-DNA Ab in Tg Mice

The impaired responses of Tg B cells to T-dependent Ag stimulation prompted us to assess the general status of the humoral compartment in CD4C/HIV^{MutA} Tg mice. Total IgG_1, IgG_{2a}, and IgG_{2b} levels were significantly decreased in the serum of Tg mice from different age groups as compared to non-Tg controls (Table 1 and data not shown). Total serum IgM levels tended, however, to be higher in Tg mice than in non-Tg controls. IgE levels were comparable in sera from non-Tg and Tg mice. This pattern of IgG production further exemplifies the impaired ability to obtain switched Ab isotypes in Tg mice. Furthermore, the data suggest an aberrant control of IgM production in Tg mice, possibly resulting in autoimmune dysfunction.

Indeed, when the sera of Tg mice were assessed for the presence of auto-Ab, animals with significant disease (2.5- to 5.5-month-old) were found to have an increase in anti-DNA IgM titers (Figure 4B). Consistently, only low anti-DNA Ab levels of the IgG isotypes could be detected, in contrast to that found in the serum of control NZB mice. Despite the increased levels of auto-Ab in Tg animals, FACS analysis revealed no more $CD5^+$ B cells in the spleen, LN, and peritoneum of Tg than non-Tg mice (data not shown).

Peripheral B Cell Expansion in Tg Mice Is Not Associated with Increased Bone Marrow (BM) Precursors

An increase of ~25%–50% in absolute B cell numbers was observed in the spleen and LN of CD4C/HIV^{MutA} Tg mice (Hanna et al., 1998b; Figures 5 and 6). Additional studies have shown that the numbers of both low-density and high-density small resting B cells are elevated (data not shown). We have therefore assessed the status of B cell precursors in the BM of Tg mice. The number of $CD43^+$ $B220^+$ pro-B and IgM^+ $B220^+$ immature B cells were not increased when compared to those of non-Tg mice (data not shown), suggesting that the expansion of the mature B cell compartment is favored by factors outside the BM.

B Cells from Tg Mice Are Not Impaired in Their Capacity to Isotype Switch In Vitro

In order to further clarify the status of B cells in the Tg mice, the phenotype and function of purified small resting B cells were investigated. FACS analysis revealed that Tg B cells isolated from the LN and spleens of mice of different age groups showed no change in the levels of surface expression for IgM, MHC class I, and CD40 as compared to B cells from non-Tg littermates (data not shown). Only in older animals was a slightly diminished level of MHC class II observed. Resting Tg B cells did not express altered levels of CD25, CD44, CD69, CD80, or CD86 on their surface when compared to non-Tg B cells (data not shown). In vitro analysis of B cell function showed similar amounts of cells producing IL-10 in response to lipopolysaccharide (LPS) in non-Tg and Tg mice (data not shown). However, Tg B cells proliferated spontaneously to a slightly greater extent than non-Tg B cells and had a slightly elevated response to CD40 stimulation (Figure 4C). Also, secreted IgM levels were found to be slightly higher in the supernatants of unstimulated Tg B cells than that of non-Tg B cells (Figure 4D). Since Tg B cells proliferated ~1.5-fold better in vitro than non-Tg B cells, the modest increase of IgM levels observed appeared to reflect a greater number of cells producing these molecules rather than a higher number of molecules produced per cell. Total IgM and IgG_1 production by Tg B cells following in vitro treatment with LPS or anti-CD40 plus IL-4 was found to be comparable to that of non-Tg B cells (Figures 4D and 4E). Levels of apoptosis in Tg versus non-Tg B cell cultures were similar as assessed by FACS analysis with annexin V and propidium iodide labeling (data not shown). Therefore, given their capacity to Ig isotype switch following in vitro stimulation, it seems that some anomalies of Tg B cells are not intrinsic but rather involve a disturbed in vivo environment.

The Development of the AIDS-like Disease in Tg Mice Does Not Require IFN-γ

Since human AIDS is associated with high levels of IFN-γ, including increased production by $CD4^+$ T cells (Graziosi et al., 1996; Emilie et al., 1994; Than et al., 1997), and given that a higher proportion of $CD4^+$ T cells from Tg versus non-Tg mice produce IFN-γ following in vitro stimulation, the role of this cytokine in the development of the AIDS-like disease was investigated. CD4C/HIV^{MutA} Tg mice bred to an IFN-γ gene-deficient background (IFN-$\gamma^{-/-}$) still developed an AIDS-like disease with a slightly faster progression to death (Figure 5A). Gross and histopathological observations were indistinguishable from that of wild-type (Hanna et al., 1998b) or of heterozygote (IFN-$\gamma^{+/-}$) Tg mice (Figures 5D and 5E). These double mutant mice also progressively lost their $CD4^+$ T cells, accumulated higher B cell numbers (Figure 5C), and produced anti-DNA IgM (Figure 5B).

with sense (H) or antisense (I) probes. Spleens from non-Tg (L) or Tg (M) mice were exposed with an antisense probe. (J, K, N, and O) are bright field images of the same regions shown in dark field in (H, I, L, and M), respectively. The scale bar in (H) represents 500 μ and is valid for (H–K). The scale bar in (L) represents 1000 μ and is valid for (L–O). Counterstain, H&E. SC, subcapsular sinus.

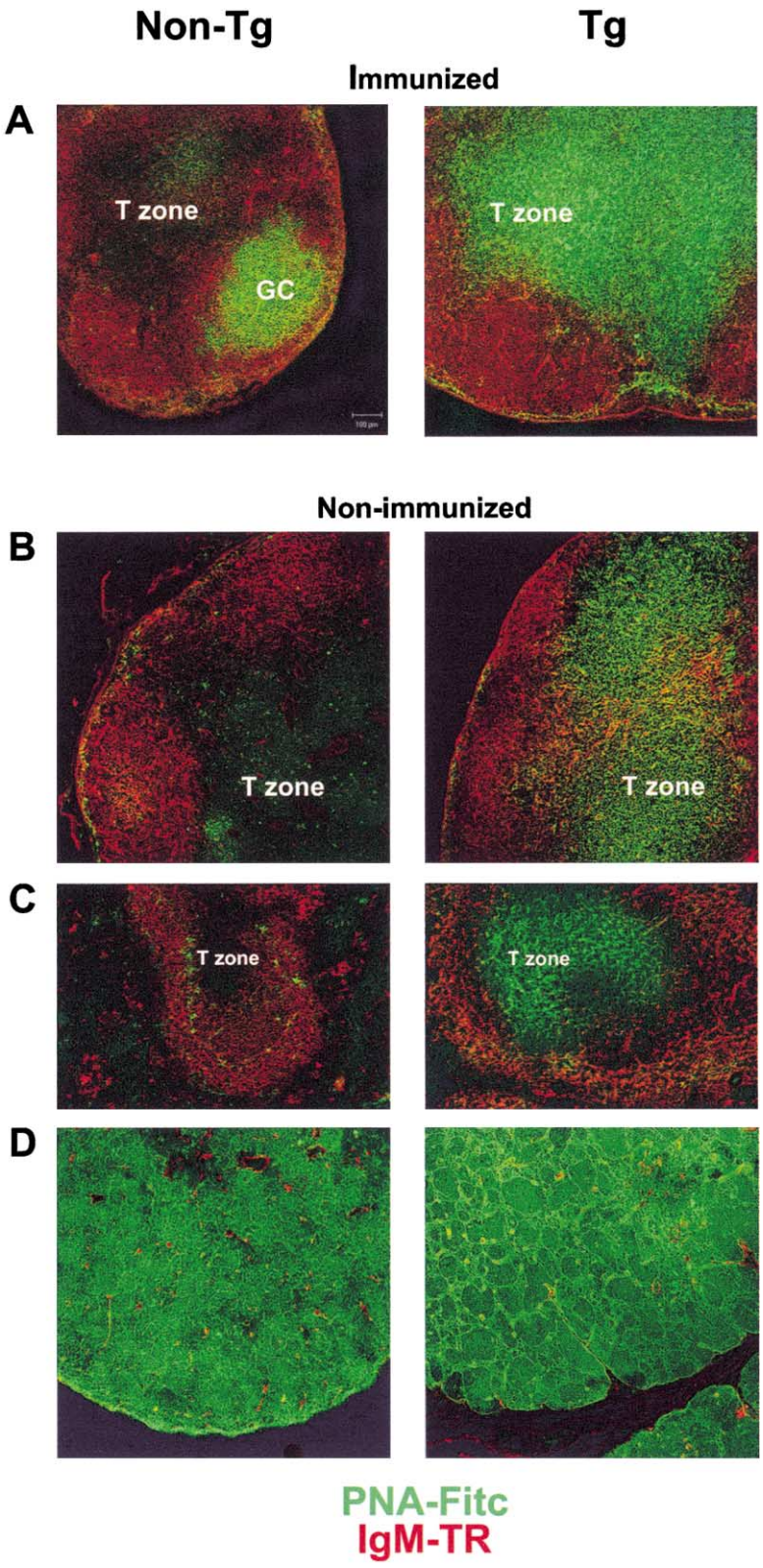

Figure 3. Strong PNA Binding in the T Zone of Tg Lymphoid Organs

OVA-immunized (A) and nonimmunized (B–D) non-Tg and Tg mice were studied. PNA binding (green) and IgM expression (red) levels and distribution in cryosections of LN taken at day 10 following primary immunization with OVA (A). Cryosections of LN (B), spleen (C), or thymus (D) of nonimmunized mice. Sections were analyzed by confocal microscopy. Data are representative of at least two experiments (eight mice).

IL-6-Deficient Tg Mice Still Develop the AIDS-like Disease

Given the importance of IL-6 in B cell responses, GC reactions, and auto-Ab production (Richards et al., 1998), and its elevated levels in a high proportion of AIDS patients (Emilie et al., 1994; Graziosi et al., 1996; Lafeuillade et al., 1991), we also assessed whether disease will develop in CD4C/HIV^{MutA} Tg mice bred on an

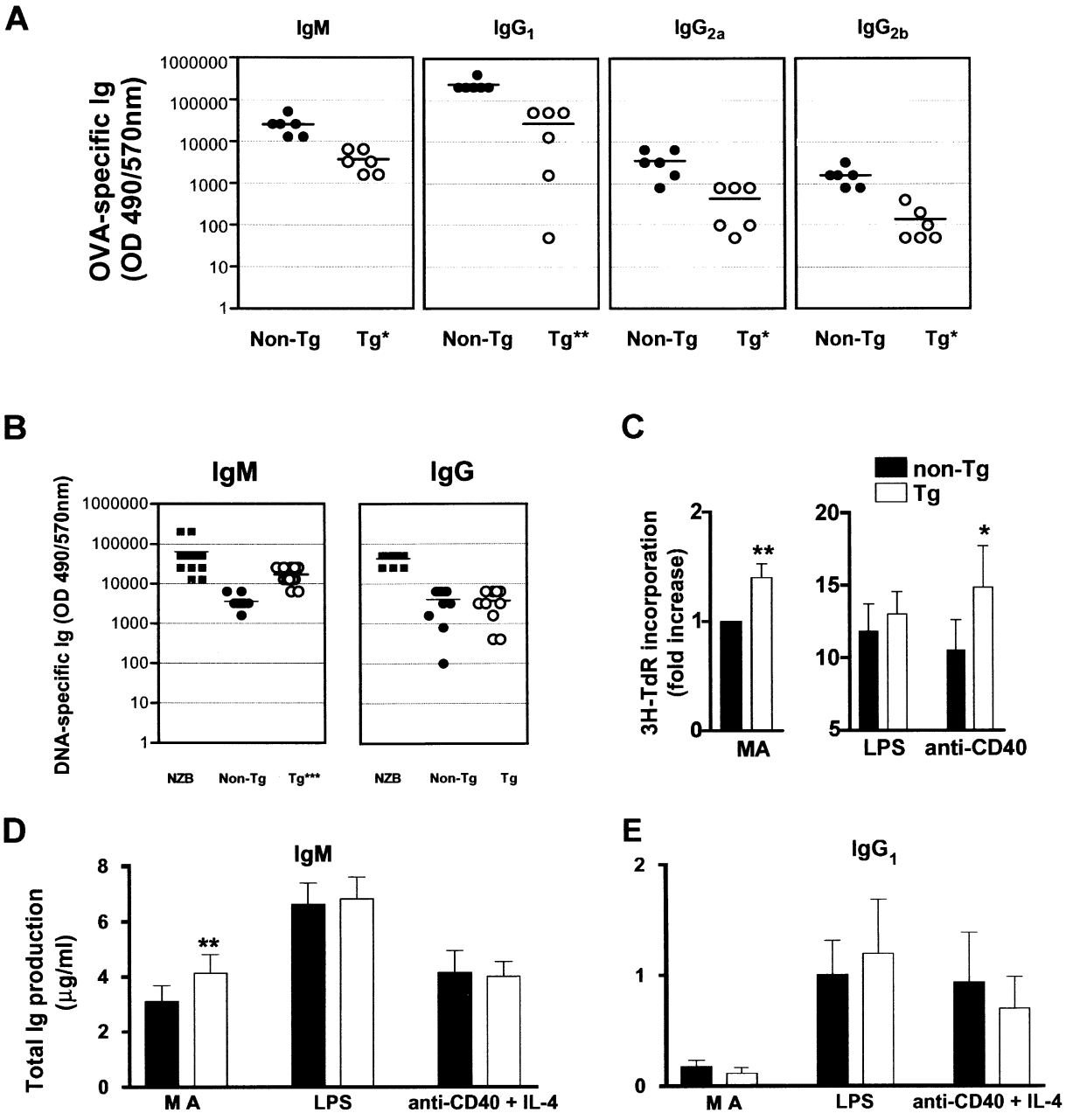

Figure 4. Reduced OVA-Specific Ig Isotype Switching, the Presence of Anti-DNA Ab, and In Vitro Function of B Cells in Tg Mice

(A) OVA-specific Ab titers in the serum of non-Tg and Tg mice taken 5 days following secondary immunization were measured by ELISA. Each point represents the serum of one mouse at the endpoint dilution of the titration curve. The bars show mean values from six different experiments.

(B) Anti-DNA IgM and IgG Ab levels in the sera of nonimmunized non-Tg and Tg mice were measured by ELISA. The data shown were normalized to the DNA reactivity observed with reference sera from three autoimmune NZB mice (arbitrarily established as 100%; filled square). Each dot represents the serum of one mouse at the endpoint dilution of the titration curve.

(C–E) Resting B cells purified from non-Tg and Tg mice were cultured with either medium alone (MA), LPS, or anti-CD40 mAb, in the presence (D and E) or absence (C) of murine IL-4. Proliferation (C) was measured by [^{3}H]thymidine incorporation (cpm). Data are presented as fold increase in response over that of the value obtained with non-Tg cells in medium alone. Total levels of IgM (D) and IgG$_1$ (E) production (μg/ml) were measured in 6-day culture supernatants. The scale bars on the graphs represent the mean of seven experiments ±SEM (n = 7). Student's t test, *p = 0.05; **p = 0.001; ***p = 0.0001.

IL-6 gene-deficient background (IL-6$^{-/-}$). IL-6$^{-/-}$ double mutant Tg mice were found to be similarly susceptible both in terms of gross and histopathology to the development of the AIDS-like disease leading to death as the heterozygote (IL-6$^{+/-}$) Tg mice (Figures 6A, 6D, and 6E). As for IL-6$^{+/-}$ Tg mice, IL-6$^{-/-}$ Tg mice exhibited a de-

Table 1. Ig Isotypes in the Serum of CD4C/HIV^{MutA} Tg Mice

	IgG1		IgG2b		IgM		IgE	
Age (months)	nTg	Tg	nTg	Tg	nTg	Tg	nTg	Tg
1–3	123 ± 16[a]	29 ± 8***	25 ± 4	9 ± 1.4***	30 ± 4	48 ± 7*	0.013 ± 0.006	0.04 ± 0.02
3–5	146 ± 28	30 ± 6**	49 ± 9	19 ± 5**	33 ± 3	45 ± 9	0.04 ± 0.015	0.04 ± 0.01
5–7	138 ± 25	43 ± 13**	34 ± 2	10 ± 2***	30 ± 2	44 ± 11	0.02 ± 0.003	0.06 ± 0.02
Total	138 ± 14	33 ± 5***	34 ± 4	13 ± 2***	30 ± 2	45 ± 5*	0.02 ± 0.006	0.04 ± 0.009

[a] Concentrations in mg/100 ml
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t test
1–3 months, n = 13; 3–5 months, n = 15; 5–7 months, n = 10 each, Tg and nTg
Data are presented by mean ± SEM.

crease in CD4$^+$ T cells, increased B cell numbers in their LN (Figure 6C), and the production of IgM anti-DNA auto-Ab (Figure 6B).

Discussion

The peculiarity of the CD4C/HIV Tg mice resides in the fact that disease development is mainly attributable to a single HIV-1 gene product Nef, occurring without viral replication and in the absence of an immune response against HIV-1 (Hanna et al., 1998b). The specific pattern of expression in target cells naturally infected by HIV-1 in humans likely explains the very high resemblance of this mouse disease to human AIDS. Furthermore, despite the fact that virtually all target cells express HIV-1 in this model, its features are likely to remain relevant to human AIDS because mosaic Tg founders or chimeric HIV Tg mice, which harbor lower numbers of HIV$^+$ cells, still develop the same AIDS-like disease, but after a longer period of latency (Hanna et al., 1998a, 1998b).

In the present analysis, we show that lymphoid pathology was accompanied by more subtle defects of immune structures and associated with B cell hyperactivity and auto-Ab production, phenomena also reported in AIDS (Edelman and Zolla-Pazner, 1989; Shearer, 1998; Root-Bernstein, 1990).

HIV-1 Impairs the Development of T Cell-Dependent GC Reactions

Our study clearly shows that the typical T-dependent GC formation and Ag-specific Ab maturation in response to immunization with OVA does not develop in Tg mice and that the FDC network is greatly reduced. One of the main causes of the impaired GC formation and reduced FDC network in these Tg mice is likely to be the lack of T cell help (MacLennan, 1994; Kosco-Vilbois et al., 1997), which is manifested in these animals by the preferential and progressive loss of peripheral CD4$^+$ T cells (Hanna et al., 1998b) and by an impaired capacity of the remaining CD4$^+$ T cells to express CD40L. CD40L plays a pivotal role in the establishment of GC reactions, isotype switching, and generation of memory. Our observations are consistent with those obtained with CD40$^{-/-}$ (Kawabe et al., 1994) and CD40L$^{-/-}$ mice (Xu et al., 1994). Although the downregulation of CD4 may contribute to the impaired T helper functions in these Tg mice, we present evidence with the Tg mice expressing a Nef^{Myr-} mutant allele that the phenotypes observed in these mice are not solely the result of CD4 downregulation. In addition, the abnormal DC phenotype and function found in CD4C/HIV^{MutA} Tg mice (J.P., unpublished data) are likely to contribute to T cell loss and impaired T cell function (Steinman et al., 1997).

The phenotypes found in CD4C/HIV^{MutA} Tg mice are characteristic of human HIV infection, where the loss of CD4$^+$ T cells is one of the most prevalent phenotypes (Shearer, 1998; Pantaleo and Fauci, 1995). CD40L expression is also impaired even after in vitro stimulation of CD4$^+$ T cells obtained from HIV$^+$-infected patients (Macchia et al., 1993; Wolthers et al., 1997; Vanham et al., 1999). Low numbers of GC and disrupted FDC networks in lymphoid organs are very prevalent features of advanced human AIDS (Tenner-Racz et al., 1986; Tenner-Racz and Racz, 1995; Pantaleo et al., 1993; Pileri et al., 1986), and are thought to be attributable to a concomitant contribution of cytopathic effects and exhaustion of the immune response. Our data suggest that the formation of GC itself and the lack of FDC networks, which limit B cell migration and accessibility to Ag, as opposed to their destruction once generated, may also contribute to the impairment observed in human AIDS.

Impaired B Cell Functions and Auto-Ab Production Caused by HIV-1

Consistent with the reduced capacity to generate GC, Ig isotype switching was decreased in response to OVA in Tg mice. This is likely to reflect impaired delivery of T cell help rather than an incapacity to switch, given that Tg B cells respond to in vitro CD40 stimulation by producing IgG in a similar fashion to non-Tg B cells. Also, although GC formation was prevented in these Tg mice, a higher proportion of large IgM$^+$ cells, possibly plasma cells, was found in both LN and spleen regardless of whether mice had been immunized, and was associated with high levels of IgM and very low levels of IgG in sera. Such elevated serum IgM levels are similar to the hyperglobulinemia reported in AIDS (Shearer, 1998; Edelman and Zolla-Pazner, 1989). In human AIDS, the accumulation of IgM in sera is present but not prevalent (Moodley et al., 1997; El-Sadr et al., 1984), most likely because GC formation and isotype switching can still occur, in contrast to that found in Tg mice. The consequences of impaired GC reactions in human AIDS are reflected by the reduced capacity to generate efficient neutralizing Ab and to produce and maintain mature high-affinity memory responses as the disease progresses (Pantaleo and Fauci, 1995; Shearer, 1998; Koopman and Pals, 1992).

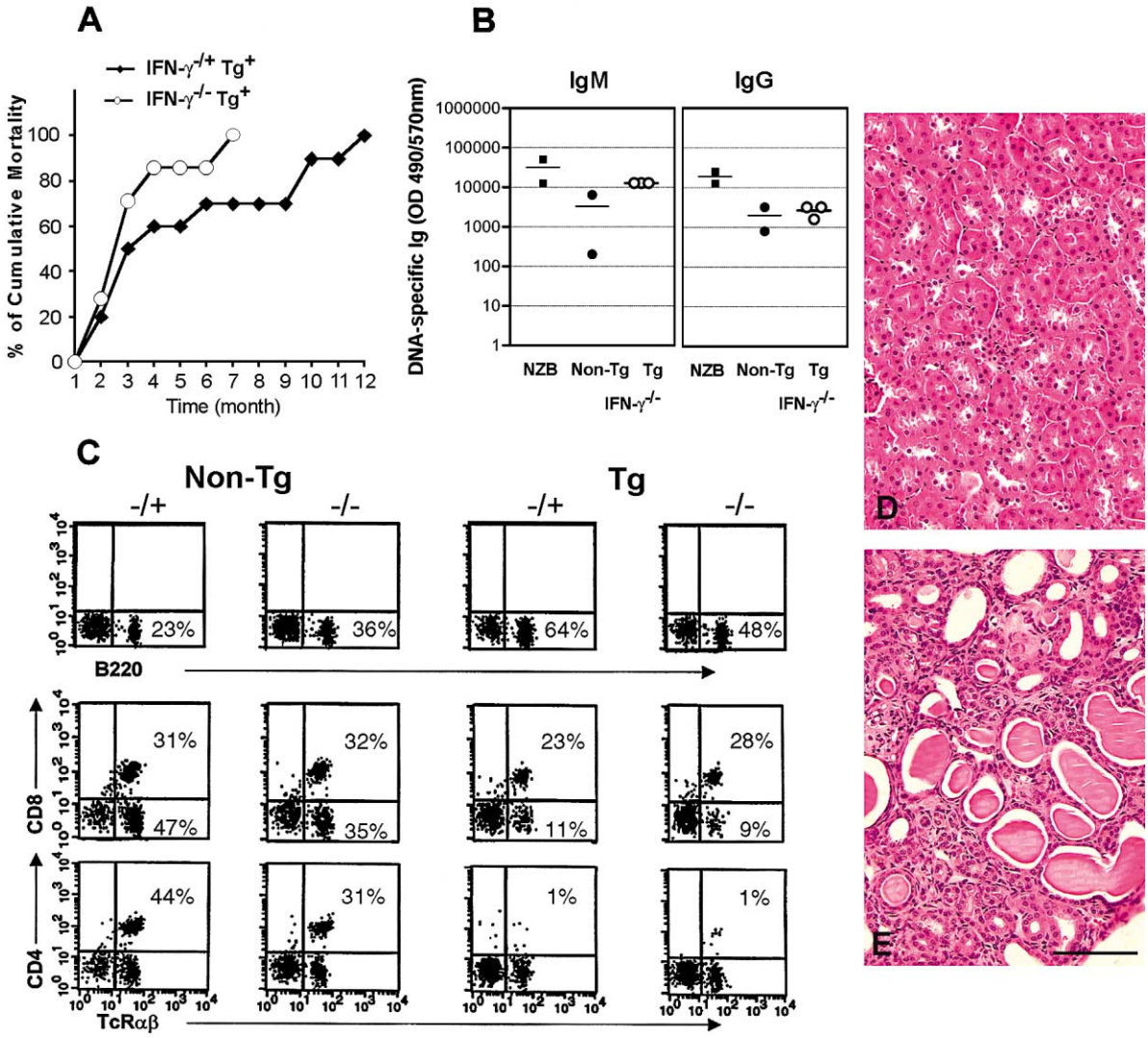

Figure 5. Tg Mice Deficient for IFN-γ Develop the AIDS-like Disease

(A) The incidence of mortality of CD4C/HIV^{MutA} Tg mice bred on homozygote IFN-$\gamma^{-/-}$ background (n = 10) is shown in comparison with that of Tg mice bred on a heterozygous IFN-$\gamma^{-/+}$ background (n = 7).

(B) The presence of anti-DNA IgM and IgG Ab in the sera of nonimmunized non-Tg and Tg IFN-$\gamma^{-/-}$ mice was measured by ELISA, as described in the legend to Figure 4. Data are representative of at least three experiments.

(C) FACS analysis shows expression of B220, CD8, and CD4 in the LN of nonimmunized non-Tg and Tg IFN-$\gamma^{-/-}$ and IFN-$\gamma^{-/+}$ mice.

(D and E) Kidney histology of IFN-$\gamma^{-/-}$ non-Tg (D) and Tg (E) mice is shown. The scale bar in (E) represents 100 μ.

The impairment of efficient control mechanisms in the generation of Ab responses in Tg mice could lead to autoimmunity. Indeed, another important characteristic of the AIDS-like disease in Tg mice is the production of auto-Ab, which are predominantly of the IgM isotype, consistent with the impaired capacity to switch found in these mice. Therefore, as in human AIDS, where auto-Ab are also frequently detected (Pantaleo and Fauci, 1995; Shearer, 1998; Root-Bernstein, 1990; Ditzel et al., 1994), the present Tg model exhibits immunodeficiency and elements of autoimmunity, both phenotypes arising in the absence of IL-6, whose levels are known to be high in human AIDS (Emilie et al., 1994; Lafeuillade et al., 1991; Graziosi et al., 1996).

The accumulation of auto-Ab and the high IgM levels in the sera of Tg mice are likely to reflect the higher number and probably the state of activation of Tg B cells, which were found to proliferate spontaneously in vitro and to a greater extent than non-Tg B cells following stimulation. Given that Tg B cells do not express HIV-1, further experiments will be required to determine whether this B cell hyperreactivity is the result of aberrant signals delivered by the in vivo environment and/or whether a subpopulation of B cells is selectively activated and/or expanded in these mice.

Interestingly, B cell activation is also characteristic of human AIDS, in the early phase of the disease (Pantaleo and Fauci, 1995; Shearer, 1998; Koopman and Pals, 1992; Edelman and Zolla-Pazner, 1989). Expansion of B cell immunoblasts and increased IgM production have also been seen to occur in another HIV Tg model, although in direct contrast to our observations, these Tg

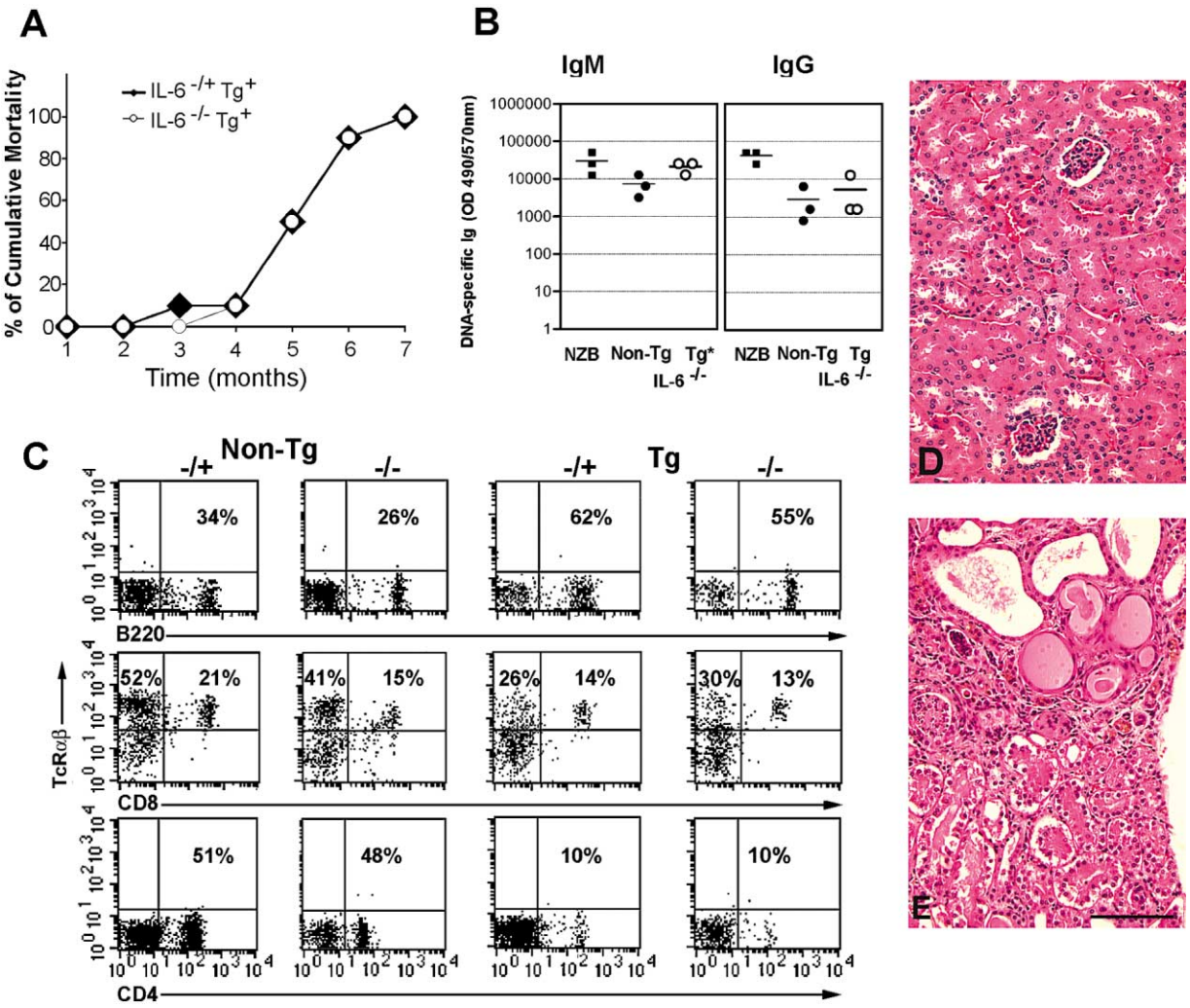

Figure 6. Tg Mice Deficient for IL-6 Develop the AIDS-like Disease

(A) The incidence of mortality of CD4C/HIV^{MutA} Tg mice bred on a homozygous IL-6$^{-/-}$ background (n = 10) is shown in comparison with that of Tg mice bred on a heterozygous IL-6$^{-/+}$ background (n = 10).

(B) The presence of anti-DNA IgM and IgG Ab in the sera of nonimmunized non-Tg and Tg IL-6$^{-/-}$ was measured by ELISA, as described in the legend to Figure 4. Data are representative of at least three experiments. Student's t test, *$p < 0.05$.

(C) FACS analysis shows expression of B220, CD8, and CD4 in the LN of non-immunized non-Tg and Tg IL-6$^{-/-}$ and IL-6$^{-/+}$ mice.

(D and E) Kidney histology of IL-6$^{-/-}$ non-Tg (D) and Tg (E) mice is shown. The scale bar in (E) represents 100 μ.

mice were thought to have expanded GC in their spleen (Tinkle et al., 1997a). However, in these Tg mice, the interpretation of the lymphoid organ phenotype might be confounded by the simultaneous presence of severe and widespread vasculopathy leading, among other changes, to splenic infarcts and extensive cell death (Tinkle et al., 1997b).

Since B cells are activated and accumulate in CD4C/HIV^{MutA} Tg mice in the context of immunodeficiency, it would be expected that B cell lymphomas would arise at a high frequency. This is intriguingly not the case, although B cell lymphomas are frequently associated with human AIDS (Koopman and Pals, 1992; Herndier et al., 1994), and are known to be frequently accompanied by auto-Ab production (Herndier et al., 1994). The expansion of the MZ that we observe in Tg mice with its associated B cells may be the first step in the development of such lymphomas, although MZ B cell-derived low-grade MALT lymphomas only occasionally develop in AIDS (Teruya-Feldstein et al., 1995). The fact that fully malignant B cell lymphomas do not arise in our Tg mice could be due to an unfavorable genetic background. Alternatively, the absence of B cell lymphoma may reflect the biology of the system, especially the absence of GC-derived B cells in Tg mice, which may constitute the target cells for transformation. In fact, most AIDS-associated B cell lymphomas are thought to originate from memory B cells and are mostly the result of immune dysregulation (Koopman and Pals, 1992; Herndier et al., 1994).

Unusual Lymphoid Structure and Expansion of the Splenic MZ in CD4C/HIV-1^{MutA} Tg Mice

Our immunohistochemical analysis revealed that the Tg T cell zone was remarkable by its abnormally strong PNA binding, both in OVA-immunized and nonimmunized Tg

mice, apparent mostly on cells with lymphoid morphology and in larger cells with macrophage/dendritic morphology. PNA has a strong affinity for GC B cells (Kosco-Vilbois et al., 1997), as well as for normal thymocytes (Wu et al., 1997) and activated peripheral effector/memory $CD4^+$ and $CD8^+$ T cells (Galvan et al., 1998). Interestingly, HIV-1-infected human $CD4^+$ T cells and macrophages have also been shown to bind strongly to PNA (Lefebvre et al., 1994; Perrin et al., 1997). However, the biological meaning of this strong PNA binding in Tg mice remains unclear.

Strikingly, we found an enlargement of the splenic MZ in OVA-immunized Tg mice. The normal MZ is a specialized structure containing mainly immature $IgM^{hi}IgD^+$ B cells as well as memory B cells, macrophages, and DC that are thought to have an important role in the generation of T cell-independent Ab responses (Witmer and Steinman, 1984; Cyster, 2000). In the Tg mice, the enlarged MZ contains numerous IgM^{hi} B cells and numerous macrophages and DC (J.P., unpublished data) which contribute to the large nonlymphoid PNA binding cells in Tg MZ. Expansion of the splenic MZ is a rare phenotype and has been recently reported in BLC-deficient mice (Ansel et al., 2000) and to be associated with the overexpression of BlyS, which also leads to B cell hyperactivity, decreased numbers of T cells, and the development of an SLE-like autoimmune disease (Moore et al., 1999; Mackay et al., 1999; Cyster, 2000; Khare et al., 2000), a phenotype resembling that of $CD4C/HIV^{MutA}$ Tg mice. Therefore, expansion of the MZ and generation of anti-DNA IgM in these Tg mice may be induced by factors secreted by HIV-1-expressing MZ macrophages or DC, such as BlyS, which is expressed by monocytes, macrophages, and DC (Cyster, 2000). Interestingly, the fact that most macrophages and DC express the HIV-1 transgene in these mice may have helped to unravel this expanded MZ structure, which may not be easily recognizable when few cells express HIV-1 gene products, as in HIV-1-infected individuals.

Finally, our immunohistochemical studies revealed the presence of large PNA^{hi} binding cells in the surroundings of large IgM^+ cells which are probably plasma cells, both cell types being increased in the red pulp of Tg mice. This cell association could reflect an ongoing activation process leading to plasma cell differentiation and survival.

Development of the AIDS-like Disease in $CD4C/HIV^{MutA}$ Tg Mice Does Not Require IFN-γ

It has been proposed, but not confirmed, that human AIDS development is associated with a Th1 to Th2 cytokine shift (Clerici and Shearer, 1994; Graziosi et al., 1994), although a role for these cytokines in progression to AIDS has yet to be elucidated. We found that a higher percentage of Tg than non-Tg $CD4^+$ T cells were capable of producing IFN-γ following in vitro activation, a finding consistent with the high levels of IFN-γ reported in human AIDS, including in $CD4^+$ T cells (Graziosi et al., 1994, 1996; Emilie et al., 1994; Than et al., 1997). The increased IFN-γ-producing Tg $CD4^+$ T cells might reflect a direct effect of HIV-1 and may be an adaptive response to favor virus production, since IFN-γ has been shown to activate HIV-1 expression (Koyanagi et al., 1988). Also, HIV-1 may favor an outgrowth and/or selection of a T cell subpopulation, and in fact, the existence of $CD4^+$ T cell subpopulations with regulatory properties has already been demonstrated (Saoudi et al., 1996). The finding that $CD4C/HIV^{MutA}$ Tg mice bred onto an IFN-$\gamma^{-/-}$ background have a slightly faster progression to death than their heterozygote littermates may support a regulatory role for IFN-γ in disease progression. However, IFN-γ itself is clearly not essential for the development of an AIDS-like disease or production of auto-Ab in Tg mice.

Conclusions

The numerous phenotypes observed in the $CD4C/HIV^{MutA}$ Tg mice are very similar to those found in human AIDS, suggesting that they may share similar underlying cellular and molecular causes. We have shown that HIV-1 can modulate expression of surface molecules and affect cellular functions, contributing to the generation of an impaired environment which can lead to dysregulated interactions and breakage of peripheral tolerance, allowing for the establishment of immunodeficiency and the potential for autoimmune disease.

Experimental Procedures

Mice and Immunization

The $CD4C/HIV^{MutA}$ Tg mice have been previously described (Hanna et al., 1998b). The CD4C/HIV-Nef^{Myr-} DNA has been constructed by site-directed mutagenesis (G→A, position 2) of the myristilation site. The mutated *nef* sequences were reintroduced in the $CD4C/HIV^{MutG}$ DNA backbone (Hanna et al., 1998b) replacing the wild-type *nef* of HIV-1. Tg mice were generated and founder lines expressing equal and higher levels of Nef than the $CD4C/HIV^{MutG}$ Tg mice were established (Z.H., unpublished data).

Sex-matched non-Tg and Tg littermates were used between 6 and 20 weeks of age. The IFN-$\gamma^{-/-}$ (Dalton et al., 1993), and IL-$6^{-/-}$ (Kopf et al., 1994) mice were obtained from the Jackson Laboratories. Animals were kept under SPF conditions. Mice (8–10 weeks of age) were immunized with alum-precipitated OVA (Sigma) as described previously (Poudrier et al., 1999).

Ab and Reagents

Ab specific for B220 (RA36B2), CD4 (GK1.5), CD8 (53.6.78), Thy 1.2 (30H12), CD3ϵ (145-2C11), and MHC class II (M5-114) were from the ATCC. The anti-FDC-M2 (209) mAb has been described (Kosco-Vilbois et al., 1997). Anti-CD69, -CD44, -CD45Rb, -Mac-1, -IA^k, -B7-1, and -B7-2 were from Cederlane. The Pan-NK cell DX5, anti-CD5.1, and anti-CD40L mAb were from Pharmingen. Goat anti-mouse IgM-Texas-red, Cy5-streptavidin, and FITC-streptavidin were from Southern Biotechnology Associates. Mouse anti-rat IgG F(ab′)2-FITC was obtained from Jackson Immunoresearch Laboratories. Biotinylated PNA was from Vector. Anti-CD40 (FGK-45) was from Dr. A. Rolink (Basel Institute for Immunology, Switzerland). Anti-CD28 (37.51) was from Dr. P. Hugo (Procrea, Canada). Mouse recombinant IL-4 was from the X63-mIL-4-transfected plasmacytoma (Karasuyama and Melchers, 1988).

Isolation of T and B Cells

High-density cells from LN or spleen of nonimmunized Tg or non-Tg mice were isolated on Percoll (Pharmacia) gradients. Further purification involved the depletion of contaminating cells using magnetic beads (Dynal) following preincubation with hybridoma supernatants. This resulted in >95% $CD4^+$ T cells or $B220^+$ B cells.

In Vitro $CD4^+$ T Cell and B Cell Activation

Cells were cultured in Iscove's modified Dulbecco medium supplemented with 5% FBS, L-glutamine (Gibco BRL), 2-ME, and antibiotics. For stimulation, $CD4^+$ T cells were cultured on anti-CD3 (10 μg/ml)-coated plates in the presence of anti-CD28 (25% supernatant). B cells were cultured with either LPS (Sigma; 10 μg/ml) or anti-

CD40 (25% supernatant), in the presence or absence of IL-4 (25% supernatant). Proliferation was measured by [^{3}H]thymidine (1 μCi/well; Amersham) incorporation for the final 18 hr of a 48-hr culture period.

Ig were detected by standard ELISA procedures using plates coated with either OVA (1 μg/ml), goat anti-mouse IgM or IgG (1 μg/ml; Southern Biotechnology Associates), or sonicated double-stranded human placenta DNA (200–300 bp; 4 μg/ml). HRP-coupled goat anti-mouse IgM, IgG1, IgG2a, IgG2b, and IgE Ab (Southern Biotechnology Associates) were used to reveal isotypes. OPD (Sigma) was used as a substrate.

Immunofluorescence

Serial cryosections of LN, spleen, or thymus were fixed in acetone and stored at −20°C. For immunolabeling, sections were rehydrated in PBS and incubated with Ab. Slides were mounted with Mowiol (Hoechst Aktiengesellschaft), and analysis was performed by fluorescent (Zeiss) and confocal microscopy (Zeiss LSM 510).

Flow Cytometry

Immunolabeling was assessed with a FACScan (Becton Dickinson). Intracellular cytokine staining was performed with the Golgiplus kit (Pharmingen).

Histology and ISH

Tissues fixed in PBS-buffered 4% formaldehyde or fresh frozen were processed for histology and ISH, using HIV-1-specific riboprobes, as previously described (Hanna et al., 1998b).

Statistics

Statistical analyses were performed according to Student's t test.

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